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A Test of Six Commercial ^{125}I-Labelled Digoxin Radioimmunoassay Kits

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Summary: The following 6 commercially available ^{125}I-labelled digoxin radioimmunoassay kits were tested:
A. Clinical Assays Gammacoat ^{125}I Digoxin Radioimmunoassay Kit.
B. Abbott Digoxin I 125 Imusay Kit.
C. Schwarz-Mann Digoxin AbTRAC (^{125}I) Radioimmunoassay Kit.
D. Squibb Digoxin Immutope Kit.
E. Boehringer-Mannheim Digoxin Radioimmunoassay Kit.
F. Corning Immophase Digoxin Radioimmunoassay Kit.
The Clinical Assays kit was used as the "reference" kit against which the other 5 kits were compared, as this kit had been in routine use for 15 months. These kits showed good correlation with the reference kit ($p < 0.001$). Kits A–D were tested to see if the high circulating steroids in the second half of a clinically normal pregnancy influenced kit performance. In these kits no "digoxin" level above 0.38 nmol · liter^{-1} was recorded, despite the high cortisol (712–1584 nmol · liter^{-1}) and total oestriol (69–800 nmol · liter^{-1}) levels in serum.
When serum from 49 hospitalised patients not under digoxin therapy were measured with the same 4 kits, one kit gave 5 false positive results (0.52–0.90 nmol · liter^{-1}), whereas the other 3 gave results below 0.52 nmol · liter^{-1}.
In another 49 hospitalised patients undergoing regular digoxin therapy, the greatest discrepancy between these 4 kits in measuring digoxin in a single serum was 1.15 nmol · liter^{-1}.
In the kits employing antibody-coated tubes, modification of procedure often led to improved sensitivity and therefore to better precision in the lower concentration range.

Untersuchungen von sechs kommerziellen ^{125}J-markierten Digoxin-Kits

Zusammenfassung: Es wurden folgende kommerziell erhältlichen ^{125}J-Digoxin-Radioimmunoassay-Kits auf ihre Vergleichbarkeit untersucht:
A. Clinical Assays Gammacoat ^{125}I Digoxin Radioimmunoassay Kit.
B. Abbott Digoxin I 125 Imusay Kit.
C. Schwarz-Mann Digoxin AbTRAC (^{125}I) Radioimmunoassay Kit.
D. Squibb Digoxin Immutope Kit.
E. Boehringer-Mannheim Digoxin Radioimmunoassay Kit.
F. Corning Immophase Digoxin Radioimmunoassay Kit.
Der Clinical Assays Kit diente als „Referenz"-Kit, da dieser bei uns bereits 15 Monate lang in der Routine angewandt wurde und damit die größten Erfahrungen vorlagen.
Die Korrelationen der Ergebnisse der 5 Kits war gut, ($p < 0.001$). Die Kits A–D zeigten keine klinisch bedeutungsvolle Kreuzreaktion („Digoxin"-Spiegel $\leqslant 0.38$ nmol · liter^{-1}) mit hohen Konzentrationen von Cortisol (712–1584 nmol · liter^{-1}) und Gesamt-Oestriol (69–800 nmol · liter^{-1}) wie sie in der 2. Schwangerschaftshälfte im Serum vorkommen.
Im Gegensatz dazu zeigte ein Kit 5 falsch-positive Werte (0.52–0.90 nmol · liter^{-1}) in Seren von 49 Patienten ohne Digoxinbehandlung. Die übrigen 3 Kits erbrachten Ergebnisse unter 0.52 nmol · liter^{-1}. Bei weiteren 49 Patienten unter Digoxinbehandlung wurden mit diesen 4 Kits z.T. erheblich unterschiedliche Werte gemessen. Die größte Differenz betrug 1.15 nmol · liter^{-1}.
Durch Modifikation der Kit-Vorschriften konnte die Empfindlichkeit, und damit die Präzision im unteren Meßbereich, der drei Coated-Tube-Assays verbessert werden.

Introduction

With the rapidly expanding use of commercial test kits, a study was undertaken to compare six digoxin radioimmunoassay kits, all using a ^{125}I-labelled digoxin derivative as tracer. The kits were tested for quality and reproducibility.

Four kits using differing techniques were further tested for interfering substances in the following three clinical situations:

a) In the second half of a clinically normal pregnancy where circulating steroids are raised.
b) In hospital patients not undergoing digitalis therapy, but receiving normal pharmaceutical preparations.
c) In hospital patients receiving regular digoxin therapy.

Finally, the three kits using the coated tube technique were tested and modified to improve sensitivity and reproducibility in the range under 1.28 nmol liter^{-1} digoxin.

Materials and Methods

The kits tested were from the following manufacturers:

A. Clinical Assays Gammacoat ^{125}I Digoxin Radioimmunoassay Kit (Clinical Assays, 217, Binney St., Massachusetts, USA).
B. Abbott Digoxin I 125 Imusay Kit (Abbott Laboratories, North Chicago, Illinois, USA).
C. Schwarz-Mann Digoxin Ab-TRAC (^{125}I) Radioimmunoassay Kit. (Schwarz-Mann, Orangeburg, New York, USA).
D. Squibb Digoxin Immutope Kit (E. R. Squibb and Sons Inc., Princeton, New Jersey, USA).
E. Boehringer-Mannheim Digoxin Radioimmunoassay Kit (Boehringer-Mannheim GmbH Diagnostika, D-8132 Tutzing FRG).
F. Corning Immo Phase Digoxin Gamma Kit (I. M. A. GmbH, D-6300 Gießen, FRG).

Tables 1 and 2 show the contents of each kit and the assay procedure as given by the manufacturer.

In order to help assess sensitivity of each kit, an additional standard of 0.26 nmol · liter^{-1} was made up from digoxin (Boehringer-Mannheim) in digoxin-free pool serum.

Serum cortisol was assayed with the Clinical Assays Gammacoat ^{125}I Cortisol Radioimmunoassay Kit using the protocol issued.

Tab. 1. Schematic representation of kit contents.

Kit	Antibody	Standards	Number	Range [nmol · liter^{-1}]	Tracer	Tracer/100 Tests [kBq]	Package Size (Tests)
Clinical Assays	Coated Tubes	Serum ready to use	4	0.64–5.12	In Buffer ready to use	74.3	50/500
Abbott	In Buffer ready to use	Serum ready to use	5	0–5.12	In Buffer ready to use	37.2	100
Schwarz-Mann	Coated Tubes	Serum ready to use	6	0–6.40	Lyophilised in tubes	55.7	100
Squibb	In Buffer ready to use	Ethanolic must be diluted	1	0–6.40	Ethanolic must be diluted	300	100/200
Boehringer-Mannheim	Coated Tubes	Serum Lyophilised	5	0–6.40	Lyophilised in Buffer Mix	223	60–80
Corning	Coated Glass Beads	Serum Lyophilised	5	0–6.40	Lyophilised with colour reagent	186	120

Tab. 2. Schematic representation of kit methodology as supplied by the manufacturer.

Kit	Serum [µl]	Buffer [µl]	Antiserum [µl]	Tracer [µl]	Incubation Time [min]	Temperature [°C]	Bound/Free Separation	Fraction Counted
Clinical Assays	50	1000	*A	100	45*F	37	Aspiration of Tube Contents	Tube (Bound)
Abbott	100	0	300	100	30	*RT	18% poly-ethylene glycol + centrifugation	Precipitate (Bound)
Schwarz-Mann	50	1000	*A	*C	60	37	Aspiration of Tube Contents	Tube (Bound)
Squibb	100	1000	50	*D	30	*RT	Charcoal + centrifugation	Supernate (Bound) + Precipitate (Free)
Boehringer-Mannheim	100	500	*A	*D	60	*RT	Aspiration of Tube Contents	Tube (Bound)
Corning	200	500*E	*B	100	30	*RT	Centrifugation + decantation	Glass Beads (Bound)

*A – Antibody-coated tubes *B – Antibody-coated glass beads *C – Tracer lyophilised in antibody-coated tubes.
*D – Tracer combined with buffer *E – Buffer + coated beads already in tubes *F – 15 min pre-incubation at room temperature before tracer addition and 37°C main incubation *RT – Room Temperature

The range covered by this kit was 0–1658 nmol · liter^{-1} using the serum standards provided.

Serum total oestriol was measured using the Amersham Oestriol ^{125}I Radioimmunoassay Kit, again using the protocol provided. Serum samples were first hydrolysed with an enzyme preparation (a mixture of β-glucuronidase and aryl sulphatase) provided in the kit. Separation of bound and free oestriol was with ammonium sulphate and the range covered by the kit was 0–1190 nmol · liter^{-1}.

Blood was taken from women attending the gynaecological outpatient clinic and who were in the second half of a clinically normal pregnancy. Blood for the other investigations was obtained from samples sent for routine analysis. In all cases, blood was taken by venesection between 07.00 and 10.00 hr.

The serum from all samples was aliquotted and frozen at – 20 °C until assay. All assays in the same experiment (e.g. pregnancy series) were carried out on the same day.

All standard curves were calculated using spline function with a Siemens 404/3 Computer system. Sensitivity was defined as the value on the standard curve corresponding to 3 standard deviations from the zero standard (%B_o) and also in nmol · liter^{-1}.

In the assays employing antibody coated tubes modifications to the assays were made to improve sensitivity and reproducibility.

Kinetic studies were carried out with one kit in order to develop an emergency assay for digoxin, giving results inside one hour from receipt of heparinised blood.

Results

Kit performance

Each kit was tested with the protocol sent by the manufacturer and the sensitivity, 50% intercept and intra-assay variation determined. In the case of the kits from Abbott, Boehringer-Mannheim and Schwarz-Mann, there were changes either in the protocol or production method and results from kits employing the changed method reported (tab. 3).

Table 4 shows the modifications made to the three coated tube assays and their effect on sensitivity and reproducibility. Each manufacturer was allotted two pool-sera, one with low- and one with high digoxin levels. The sera were so collected that the values were similar in each of the low-range and each of the high-range pools.

Tables 3 and 4 show that a pre-incubation step at low digoxin concentrations increases precision and often reduces the value of the 50% intercept. At higher digoxin concentrations, pre-incubation has little or no effect on precision.

Development of an emergency assay (Clinical Assays kit)

The aim here was to develop a quick and reliable method for measuring digoxin concentrations in patients who were either unconscious through an unknown drug overdose, or in whom the question of overdigitalisation arose.

Figure 1 shows the kinetics under three different conditions on the maximum binding of tracer in the presence of a digoxin-free serum. All three assays were pre-incubated for 7 minutes at the same temperature as the main incubation, before tracer was added to the tubes.

The final form of the emergency assay was as follows:

Blood was taken into heparinised tubes and sent to the laboratory as quickly as possible where it was centrifuged and the plasma removed for assay.

Standards were set up in singlicate to cover the range 0–10.24 nmol liter^{-1}. Two quality control sera were also set up in singlicate and the patient plasma in duplicate.

Tab. 3. Sensitivity and precision of kits using the protocol given.
The figures in brackets show where kits with different protocol or production techniques have been investigated.

Kit		Sensitivity		50% Intercept		Intra-assay precision		
		B_o [%]	[nmol · liter^{-1}]	[nmol · liter^{-1}]	N	mean [nmol · liter^{-1}]	SD	CV [%]
Clinical Assays		89.8	0.31	1.40	30	1.80	0.08	4.93
					30	4.64	0.37	7.97
Abbott	(1)	95.1	0.42	1.69	20	1.37	0.07	5.10
					20	3.60	0.15	4.16
	(2)	95.2	0.31	1.32	20	1.28	0.05	3.70
					20	3.73	0.11	2.85
Schwarz-Mann	(1)	93.8	0.58	2.74	20	0.86	0.12	13.3
					20	4.27	0.18	4.28
	(2)	78.6	0.80	2.97	20	1.00	0.22	21.9
					20	4.63	0.30	6.57
	(3)	98.0	0.07	1.86	20	1.04	0.11	10.6
					20	4.41	0.14	3.17
Squibb	(1)	98.1	0.04	1.73	20	1.55	0.06	3.96
	(2)	95.8	0.08	0.63	20	4.50	0.26	5.78
Boehringer Mannheim	(1)	99.2	0.02	2.64	20	1.07	0.30	28.5
					20	5.19	0.26	4.96
	(2)	85.8	0.52	2.28	20	0.93	0.14	15.4
					20	6.20	0.56	9.03
Corning		95.7	0.41	2.25	20	0.97	0.05	4.64
					20	4.41	0.37	8.27

Tab. 4. Modification to coated-tube kits showing sensitivity, precision and changes made.

Kit	*Method	Sensitivity		50% Intercept		Intra-assay Precision		
		B_0 [%]	[nmol · liter^{-1}]	[nmol · liter^{-1}]	N	mean [nmol · liter^{-1}]	SD	CV [%]
Clinical Assays	A	85.6	0.52	2.25	19	1.67	0.27	16.0
					19	5.00	0.20	4.00
Clinical Assays	B	99.6	0.01	0.99	28	1.51	0.05	3.41
					28	5.04	0.23	4.52
Clinical Assays	C	99.8	0.25	2.47	20	1.88	0.08	4.25
					20	5.23	0.22	4.20
Schwarz-Mann	D	95.2	0.08	1.64	22	1.00	0.08	7.27
					22	4.91	0.36	7.28
Boehringer Mannheim	E	96.6	0.18	2.17	20	1.19	0.08	6.92
					20	5.43	0.22	4.15
Boehringer Mannheim	F	90.4	0.51	3.38	14	0.92	0.15	16.5
					14	5.03	0.18	3.63

*Methods

A. No pre-incubation at room temperature before tracer addition.
B. As per protocol, but with automatic pipette.
C. Emergency method.
D. Buffer addition before serum (In protocol is the opposite).
E. Boehringer coated tubes and standards, Clinical Assays buffer and tracer Clinical Assays protocol with pre-incubation.
F. As E above but without the pre-incubation.

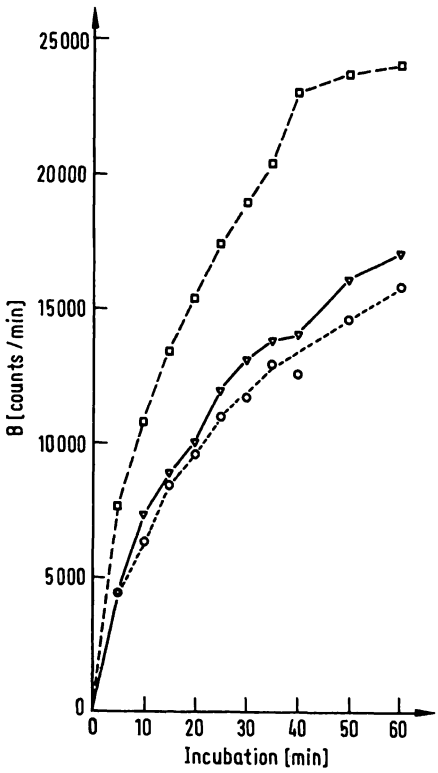

Fig. 1. Kinetic studies on the maximum binding of a zero standard at differing temperatures and tracer volumes.
o--------o 100 µl tracer, 37 °C.
Δ———Δ 100 µl tracer, 45 °C.
□————□ 200 µl tracer, 40 °C.
The abscissa represents the time in minutes from tracer addition after 7 minutes pre-incubation at the assay temperature shown in each case. The ordinate represents counts per minute bound.

1 ml Tris buffer (0.01 mol/l, pH 7.4), provided in the kit, was added to all tubes, followed by 50 µl standard/serum/plasma. The tubes were quickly vortexed and incubated for 7 minutes at 40 °C, 200 µl tracer added from a repeating syringe (Hamilton PB-600 with 10 ml syringe) and the tubes incubated with shaking for a further 20 minutes at 40 °C. The contents of the tubes were then aspirated off and the tubes washed with 2 ml Tris buffer, the contents again aspirated and the tube counted for 1 minute in a gamma counter. The standard curve was plotted by hand on semi-log paper provided with the kit and the values read off for control sera and patient plasma as soon as they were counted. If the quality control sera were within the accepted limits (inside the 2 SD range) the results of the patient sera were telephoned to the ward or doctor concerned.

The average time taken between receipt of blood and results was between 45 and 55 minutes.

The Clinical Assays kit was chosen as it was the most adaptable to such modification. Neither of the other coated tube assay systems could be used, the Boehringer kit offering no possibilities for pre-incubation, and the Schwarz-Mann kit having tracer already in the tubes.

Tests for interfering compounds

The Clinical Assays, Abbott, Schwarz-Mann and Squibb kits were tested in three differing clinical situations for possible interfering substances.

The first group of patients were 39 women in the second half of a clinically normal pregnancy. The kits were tested to see if the high steroid levels in the maternal blood affected the values obtained. All values should theoretically have been zero and in all four kits no false value above 0.38 nmol · liter^{-1} was found. The cortisol levels in the women ranged from 712–1584 nmol · liter^{-1} and were above those expected in non-pregnant women (1–3). Serum total oestriol ranged from 69–800 nmol · liter^{-1} and correlated with the week of pregnancy as expected.

The second group under study were 49 patients in hospital, not undergoing digoxin therapy, but receiving

pharmaceutical preparations. No serum was excluded from this group with the aim of including all possible interference from icteric and fatty sera as well as those with abnormal protein content. Here three kits gave no result above 0.51 nmol · liter^{-1}, but the Abbott kit showed four false positive values between 0.51 and 0.90 nmol · liter^{-1}. Serum cortisol levels in this group were significantly lower than in the pregnant women (range 36–1280 nmol · liter^{-1}, p = 0.075). Serum total oestriol was less than 25 nmol · liter^{-1} in 48 patients, the remaining value of 49 nmol · liter^{-1} occuring in a serum with low total protein, and in which the Abbott kit also gave a reading of 0.51 nmol · liter^{-1}. Both kits used a separation method which is sensitive to the globulin content of serum. The third group of 49 patients were receiving regular digoxin therapy and were used to compare performance of these four kits. In only one case did all four kits give the same result, in 29 cases two kits gave the same result, in 8 cases three kits the same and in 11 cases all kits gave different results. In 16 cases the difference between the highest and lowest values obtained for one serum was more than 0.5 nmol · liter^{-1} (the largest difference was 1.15 nmol · liter^{-1}).

Correlation between kits can be seen in table 5, using the Clinical Assays kit as the reference kit.

This group showed similar serum cortisol levels to the second group, (range 50–1208 nmol · liter^{-1}). Four patients showed serum total oestriol above 25 nmol · liter^{-1}, which corresponded with high digoxin levels from the Abbott kit and which had low total protein levels.

The correlations in Table 5 from the remaining three kits were from separate studies using routine sera for digoxin estimation. The Clinical Assays kit was used as the reference method and was run at the same time as the test kit.

The results from the Abbott kit were significantly higher than those from the Schwarz-Mann kit (*Wilcoxon Rank* Test U = 1004; p = 0.10).

Table 6 shows the results from each kit on the day tested, using both commercial and laboratory quality control sera. The same batches of control sera were used throughout the study. The values for the control sera lay in most cases within the ascribed limits, except in the Byk-Mallinkrodt sera where the values were repeatedly lower than those given. The difference in values given for the different control sera are greater in the lower range than in the higher one. Inter-assay control was only carried out for the Clinical Assays kit using the laboratory precision sera (Table 6) when, over a 4 month period, the coefficient of variation at the lower level was 12.6%, and at the higher level was 11.4%. These values were obtained from 60 assays performed by the same technician. In the other kits an inter-assay precision study was ruled out due to cost.

Tab. 5. Correlation between the 6 kits tested. All are compared with the Clinical Assays kit as reference.

Kit	Correlation Coefficient	*Regression Constants		N
	r	a	b	
Abbott	0.910	0.17	0.91	49
Schwarz-Mann	0.891	0.14	0.79	49
Squibb	0.945	0.07	0.98	49
Boehringer Mannheim	0.965	0.01	0.97	41
Corning	0.924	–0.05	0.93	43

* Regression constants for the equation y = a + bx

Tab. 6. Performance "on-the-day" of each kit with different control sera.

Control Sera	Given range Mean ± 2SD [nmol · liter^{-1}]	Kit A	Kit B	Kit C	Kit D	Kit E	Kit F
Lederle RIA-1	1.28 ± 0.51	1.34	1.15	1.79	1.54	1.28	1.40
RIA-2	3.84 ± 0.77	3.97	4.09	4.35	4.03	4.10	4.22
Byk-Mallinkrodt (N)	1.31 ± 0.38	0.77	0.77	0.92	0.87	0.96	0.90
(P)	5.58 ± 1.28	4.67	3.46	4.25	4.38	4.81	4.22
Mölter Riacon-1	*1.15	0.96	0.77	1.15	1.08	1.13	1.02
	+1.02 ± 0.26						
	**0.90 ± 0.26						
Riacon-2	*5.63	4.74	4.80	4.61	4.93	5.06	4.93
	+4.87 ± 1.28						
	**4.99 ± 1.02						
Laboratory Precision L	++1.34 ± 0.34	1.28	1.02	1.28	0.92	1.16	1.40
H	++5.63 ± 1.28	5.95	4.75	5.48	5.20	5.49	5.38

*Mean value from 8 kits tested by manufacturer. +Value given for kit A **Value given for kit C
++Inter-assay values given by Clinical Assays kit over 4 months.
Kit A – Clinical Assays. Kit B – Abbott. Kit C – Schwarz-Mann. Kit D – Squibb. Kit E – Boehringer-Mannheim. Kit F – Corning.

Tab. 7. Measured amounts of digoxin in 5 kit standards, using the materials and procedure from the Clinical Assays kit.

Standard [nmol · liter^{-1}]	Kit B	Kit C	Kit D	Kit E	Kit F
0	0.05	0.10	0	0.05	0.10
0.64	0.90	0.74	0.57	0.64	0.58
1.28	1.47	1.47	1.09	1.28	1.21
1.92	–	2.05	–	–	–
2.56	2.75	2.62	2.24	2.62	–
3.20	–	–	–	–	3.01
3.84	–	3.58	3.71	4.03	–
5.12	6.15	–	–	–	–
6.40	–	6.59	6.02	6.78	6.40

Kit B – Abbott
Kit C – Schwarz-Mann
Kit D – Squibb
Kit E – Boehringer-Mannheim
Kit F – Corning

Finally as a comparison of quality of the standards, all standards were measured in the Clinical Assays kit and the results tabulated (tab. 7). The results show good agreement in all kit standards.

Discussion

The need to determine serum digoxin levels was emphasised before the introduction of a radioimmunoassay by *Smith* et al. (4). Since then, the pharmacokinetics and bioavailability of different preparations has been adequately described, (5–11). The need for a kit which is precise and accurate throughout the whole range of digoxin levels found in the blood has been highlighted by several authors (12–15), who have described the differing response to digoxin in different clinical situations, where toxic symptoms may occur at very much lower concentrations than those normally encountered.

As in many radioimmunoassays, reports of substances causing interference have been reported for digoxin. These include digoxin analogues (16), the effect of serum proteins (17–18), and the separation technique (19), all of which can give rise to false digoxin levels (20–23). Judging from some results, this problem has still not been solved (24–25), though with the introduction of ^{125}I-labelling, many problems arising from beta-scintillation counting such as quench-effects have disappeared (26–27). The correlation between kits using ^{3}H and ^{125}I labelling is as good as between the six ^{125}I-labelled kits tested here (26–27).

In the Federal Republic of Germany (FRG), due to the decentralised nature of health care, (as in contrast to the British National Health Service, for example,) many digoxin estimations are carried out in private laboratories or small hospitals. As many of these have no facilities for the raising and testing of antisera, or for the labelling of antigens, the use of commercial kits has increased enormously. Many of these kits are imported, only a few being produced in the FRG. In the comparison of commercial kits by other authors in different clinical situations, such as acute renal failure (16), or cardiac patients on diuretics and exercise (26), large discrepancies were apparent in the results from different kits. When results from this study and another from 1974 (27) are compared, it may be deduced that an improvement of kit components has occurred over the past three years.

The use of ^{125}I-labelling has led to quicker and simpler methods and this study has shown that while in many cases the kits all deliver similar results, in other cases there are still problems, which can be overcome by relatively small changes in procedure. Oversimplification of kits can lead to unacceptably poor precision, seen here in some of the antibody-coated tube methods at low digoxin concentration. The non-optimal methodology of kits for insulin and thyrotropin (TSH) has been published elsewhere (28–29). It has been demonstrated, that a pre-incubation to reduce non-specific adsorption phenomena often increases reproducibility at low serum levels of digoxin. In the Schwarz-Mann kit, the simple reversal of addition of buffer and serum led to marked improvement in precision at low levels. The kit components are mostly very good as shown by using the Boehringer-Mannheim coated tubes and standards with preincubation and with the tracer, buffer and protocol of Clinical Assays (Tables 3 and 4). An example of the quality of the standards provided in the kits is seen in Table 7. The results from the pregnant women showed that the effect of high circulating steroid levels had little or no effect on the kits, despite the structural similarities with digoxin. In contrast, the results from the hospitalised patients not taking digoxin highlighted the probability of false results through medication and serum proteins in some kits. It was interesting to note that the two methods, namely the Abbott digoxin and Amersham oestriol kits, which used a protein-sensitive separation technique, gave false positive results in the same serum samples. The addition of carrier serum before separation in the Abbott kit, as recommended in another kit using polyethylene glycol separation (30), may very well abolish many false positive results with this kit.

In conclusion it may be said that while the quality of kit components is on average very good, major improvements can be attained by minor modification in procedure.

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